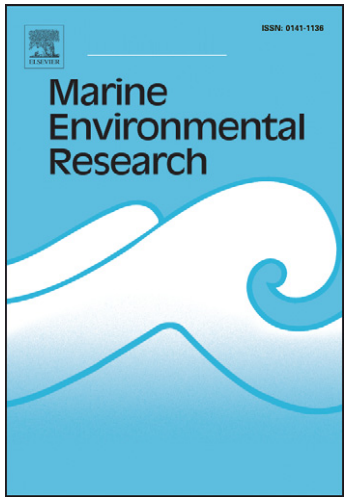


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PII: S0141-1136(08)00041-X
DOI: 10.1016/j.marenvres.2008.02.034
Reference: MERE 3193

To appear in: *Marine Environmental Research*

Please cite this article as: Chora, S., McDonagh, B., Sheehan, D., Starita-Geribaldi, M., Roméo, M., Bebianno, M.J., Ubiquitination and carbonylation as markers of oxidative-stress in *Ruditapes decussatus*, *Marine Environmental Research* (2008), doi: 10.1016/j.marenvres.2008.02.034

# Ubiquitination and carbonylation as markers of oxidative-stress in *Ruditapes decussatus*

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**Abstract**

Environmental pollutants, such as metals, are widespread in aquatic environments and can lead to the formation of reactive oxygen species (ROS). ROS are highly toxic in marine species since they can cause serious reversible and irreversible changes in proteins including ubiquitination and modifications such as carbonylation. This study aimed to confirm the potential of ubiquitination and carbonylation as markers of oxidative stress in the clam *Ruditapes decussatus* (Veneroida, Veneridae) exposed to cadmium (40 µg/L). After 21 days of

exposure clams were dissected into gills and digestive gland. Cytosolic proteins of both tissues were separated by two-dimensional electrophoresis (2-D SDS-PAGE) and analysed by immunobloting. Higher ubiquitination and carbonylation levels were in digestive gland of contaminated organisms. These results confirm the potential of ubiquitination and carbonylation as a sensitive and specific marker of oxidative stress in marine bivalves. In this approach, changes in protein structure provide options for affinity selection of sub-proteomes for 2D SDS-PAGE, simplifying the detection of protein biomarkers using proteomic approach.

*Keywords*: Reactive oxygen species; Proteomics; *Ruditapes decussates*; Ubiquination; Carbonylation

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Metals, including cadmium, can generate oxidative stress in marine molluscs (McDonagh et al., 2005; Dowling et al., 2006). Changes in protein ubiquitination and carbonylation processes are two biochemical perturbations resulting from oxidative stress (McDonagh and Sheehan, 2006). As with many marine bivalves, *R. decussatus* is able to accumulate relatively high concentrations of Cd and induce changes in other antioxidant defences, such as superoxide dismutase, catalase and decrease glutathione peroxidase activity (Bebianno and Serafim, 2003; Géret et al., 2002). Therefore, this study aimed to determine the levels of protein carbonylation and to analyse ubiquitination to investigate whether these two biomarkers of oxidative damage are affected in *R. decussatus* after Cd exposure.

Clams were collected at "Ria Formosa" lagoon (Bebianno and Serafim, 2003) and exposed to Cd (40 µg/L) for 21 days. The sub-lethal Cd concentration used was based on previous studies with the same species (Géret et al., 2002; Serafim and Bebianno, 2007). Clams were measured and dissected into gills and digestive gland. These tissues were homogenised and centrifuged (15,000 g, 2 h) and the protein content determined by the Bradford method. Aliquots of 80 µg of protein content were precipitated in cold acetone and loaded on 7 cm strips pH 3-10 NL, added to 125 µL of rehydration buffer (7M urea, 2 M thiourea, 4% CHAPS, 0.8 % Pharmalyte; 65 mM DTT, 10% isopropanol, and bromophenol blue traces). After 12h of passive rehydration, proteins were focused (250 V, 15 min; 1000 V, 30 min; 4000 V, 4h, until 20000 Vh) on a Protean IEF Cell (BioRad). Post IEF, only the strips for carbonylation were derivatized (10 mM 2,4-dinitrophenylhydrazine in 10% trifluoroacetic acid) for 20 mins and then placed in neutralisation buffer (2M Tris Base / 30% glycerol) for 20 mins. All strips were equilibrated prior to SDS PAGE, loaded on 10% polyacrylamide gels and run at a constant voltage (200V) and temperature (20ºC). Three replicates of each gel were run. Once electrophoresis was completed, gels were transferred to nitrocellulose membranes (0.2 µM) during 1 hr at 100 mA per blot. Protein expression profiles (PEPs) were visualised by silver staining (Rabilloud, 1992). Membranes were blocked 1h with 1.0 % BSA in PBS-Tween, washed and incubated with the first anti-body diluted in 1.0 % BSA (1/5000 Anti DNP by Sigma; 1/2000 Polyclonal rabbit anti-ubiquitin Dako Ref: Z0458) overnight, washed again and incubated with the second anti-body (1/1000 Polyclonal goat Anti rabbit Immunoglobulins HRP DakoCytomation 1/1000 anti-rabbit A-9169 Sigma) for 1h. Each blot was treated with chemiluminescent reagent, exposed to film, and developed. X-ray films of

immunoblots were scanned and quantification of ubiquitinated and carbonylated proteins performed with the 2D gels and blots using ImageMaster2D version 3.1 (Amersham).

PEPs in 2-D SDS PAGE gels for gill and digestive gland are different, demonstrating that intensity and pattern of protein expression change due to Cd exposure. In the corresponding immunoblots both tissues present quite distinct profiles for ubiquitination and carbonylation and between tissues (Fig. 1 and 2). In immunoblots probed with anti-ubiquitin, the level of ubiquitination was lower than that of carbonylation and both tissues presented a different pattern and intensity of spots. The apparently stronger staining in the control blots could be due to a residual non-specific binding or/and to a higher load in control samples. Ubiquitination earmarks damaged or short-lived proteins for proteolysis in the 20S core of the 26S proteasome (Davies, 2001; Friguet, 2006). The different patterns attained show that some proteins of gill and digestive gland are specifically targeted for ubiquitination in response to Cd treatment. In both tissues the ubiquitination pattern is different from the carbonylation one demonstrating that ubiquitination and carbonylation are independent (McDonagh and Sheehan, 2006). Immunoblots probed with anti-DNP revealed lower levels of carbonylation in controls (Fig. 2). In Cd-treated clams, the number of carbonylated proteins increased in both tissues and in the digestive gland were almost three-fold that of gills (214 vs 78) (results not shown). This confirms that ROS are produced in these tissues as a response to Cd exposure. Gills are in direct contact with the surrounding environment and reflect short-term metal exposure, whereas the digestive gland acts as a storage organ reflecting long-term metal exposure (Bebianno and Serafim, 2003). Phase I detoxification often results in formation of ROS (Zangar et al., 2004) and this may explain the elevated carbonylation in the digestive gland (Dowling et al., 2006). Higher ROS levels in this organ in *R. decussatus* may be due to

tissue-specific accumulation of Cd (Géret et al., 2002; Serafim and Bebianno, 2007). These results confirm the potential of ubiquitination and carbonylation as sensitive and specific markers of oxidative stress in this species.

**Acknowledgements**

S. Chora was supported by an FCT grant SFRH/BD/19477/2004.

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**Figure captions**

Fig. 1. Immunoblots of ubiquinated proteins in 2D separations of digestive gland and gill proteins from control and cadmium exposed clams.

Fig. 2. Immunoblots of carbonylated proteins in 2D separations of digestive gland and gill proteons from control and cadmium exposed clams.

Figure 1

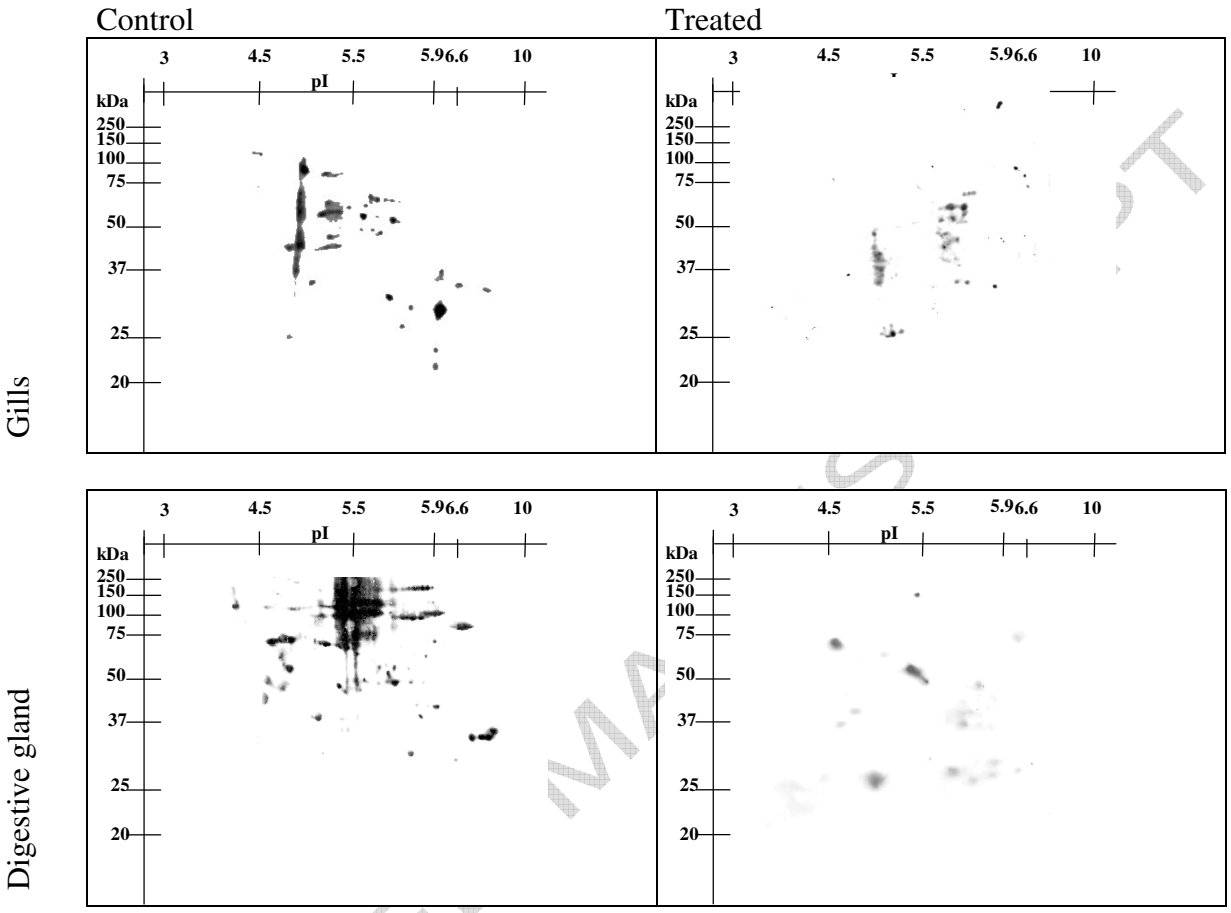

Figure 2